

EXHIBIT "A"

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of DENSLOW et al.

Confirmation No: 3958

Application No. 10/663,561

Examiner: SALMON, Katherine. D.

Filed: September 15, 2003

Group Art Unit: 1634

For: DETECTING HORMONALLY ACTIVE COMPOUNDS

37 C.F.R § 1.132 DECLARATION

Mail Stop Amendment  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

I, Nancy D. Denslow, PhD, declare as follows:

1. I am one of the named inventors and am familiar with patent application No. 10/663,561 entitled "DETECTING HORMONALLY ACTIVE COMPOUNDS" (hereafter the '561 application) and the subject matter described therein.

2. I hold a PhD degree in Biochemistry and Molecular Biology and currently am working in environmental toxicology. I am presently an Associate Professor of Physiological Sciences at the University of Florida, Gainesville, Florida.

3. I have authored or coauthored 110 scientific papers, and two issued patents (Patent #5,650,299 -- Stem Cell Proliferation Factor, Michael Lawman, Pat Lawman and Nancy Denslow.

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July 22, 1997; Patent #5,981,708 – Stem Cell Proliferation Factor, Michael Lawman, Pat Lawman and Nancy Denslow. November 9, 1999).

4. I have reviewed the Final Office Action dated September 5, 2006. I have been asked by patent counsel Zachariades to provide an explanation based on the claimed invention showing that the subject matter of the claims is not restricted to sheepshead minnow and largemouth bass fish but can be applied to other fish species without undue experimentation, unpredictability or requirement of high level of skill.

Claim 1 is copied below:

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Claim 1. A method for detecting the presence of an agent having estrogenic or androgenic activity in a sample, the method comprising the steps of:

- (A) providing at least one fish cell which was exposed to the sample;
- (B) analyzing the at least one fish cell for expression of at least one gene wholly or partially encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO's: 146, 148, 149, 166, 167, 178, 194, 199, 200, 207, 285, 347, 424, 489, 505, 509, 516, 519, 532-534, 542, 545, 551, 529 for identifying estrogen activity and SEQ ID NO's: 14, 15, 25, 28, 30, 42, 44, 47, 52, 61, 62, 71, 558 and 555 for identifying androgenic activity; and
- (C) comparing the expression of the at least one gene in the cell compared to the expression of the at least one gene in a control cell not exposed to the sample or an agent having estrogenic or androgenic activity, wherein a difference in the expression of the at least one gene in the at least one fish cell compared to the expression of the at least one gene in the control cell indicates that the sample contains an agent having estrogenic or androgenic activity.

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5. The Examiner rejects claims 1-7 and 10-32 are rejected in the instant Application in the September 5, 2006 Office Action " because the claimed invention does not reasonably provide enablement for any fish species or detection of genes partially encoded. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make or use the invention commensurate in scope with these claims."

6. I will address the Examiner's comments regarding the scope of enablement of the instant invention.

7. We describe methods of detecting expression of genes in response to agents which cause estrogenic or androgenic activity in a sample.

As discussed previously, the endocrine systems of fish are highly conserved and work by essentially the same pathways. In all fish, estrogens and androgens bind to their respective receptors to cause them to dimerize and then bind to promoter regions on genes that they control. This binding activates transcription of this set of genes. Estrogen and androgen receptors are highly conserved in sequence (for example among fish species the homology can extend up to 90%) and they regulate the same set of specific genes in different species of fish. So, that it is reasonable to expect that the same set of genes would be regulated by estrogen (and estrogen mimics) or androgen (and androgen mimics) in sheepshead minnows and largemouth bass as in

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other fish. As such, by identifying the name of the gene in these species, one of ordinary skill in the art can identify these genes in other species based on the teachings of the specification. The step-by-step methodology that can be utilized by anyone of ordinary skill in the art is based on the teachings of the invention:

**Method to Identify Homologous Sequences in other Fish Species**

1. The DNA sequence for the biomarkers we have identified in largemouth bass or in sheepshead minnow can be translated to the protein sequence by using a translation program, for example the one at the following url: <http://us.expasy.org/tools/dna.html>
2. This program will translate the DNA segment in all 6 reading frames. The correct reading frame is selected by looking for an open reading frame (between stop codons). Because the sequences were obtained by several methods, the correct reading frame may be in any one of the 6 reading frames. The correct reading frame is confirmed by using the program BLAST P that compares protein sequence to the protein databases. This program is available at the following url: <http://www.ncbi.nlm.nih.gov/blast/> and using the BLASTP program in the protein box.
3. The results of the BLAST P program identify the gene in question for all other fish species that have been deposited in gene bank, so far. The fact that genes in mammals also are identified suggests that the equivalent gene is probably in all vertebrate species.
4. If the top result is a reference to an EST in pufferfish, then one can double click the entry and then go to BLINK which will show the closest match to this EST in the entire database. Often this will show the actual name of the protein and that it is identified in multiple fish species.
5. Table 1 contains the DNA sequences and predicted protein sequences for the abbreviated list of biomarkers in the patent.
6. Fig. 1 contains examples of doing this search for three of the biomarkers on the list:
  - A. ER alpha
  - B. StAR
  - C. Spermidine/spermine acetyl transferase

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Fig. 1. Example of output from blast for two biomarkers

A. ER Alpha

Sequences producing significant alignments:

			Score	E
			(Bits)	Value
gi	42475913	gb AAG44622.2 AF253062_1	estrogen receptor alpha [Mi...	1077
gi	51100569	emb CAD43599.1	oestrogen receptor alpha [Dicentrarc...	958
gi	3122078	sp O42132 ESR1_PAGMA	Estrogen receptor (ER) (Estra...	869
gi	28192419	gb AAL82743.1	estrogen receptor alpha [Acanthopagru...	840
gi	115313958	gb AAD31032.2 AF136979_1	estrogen receptor type alp...	833
gi	85013465	gb ABC68615.1	estrogen receptor alpha [Kryptolebias...	831
gi	18996336	dbj BAB85622.1	estrogen receptor alpha [Paralichthy...	831
gi	12643248	sp Q9PVZ9 ESR1_SPAAU	Estrogen receptor (ER) (Estr...	822
gi	109804224	emb CAK95869.1	estrogen receptor type I [Oreochrom...	822
gi	3915675	sp P50241 ESR1_ORYLA	Estrogen receptor (ER) (Estra...	818
gi	31158286	dbj BAC76957.1	estrogen receptor a [Fundulus hetero...	816
gi	12230057	sp Q9YH33 ESR1_ORENI	Estrogen receptor (ER) (Estr...	815
gi	47224612	emb CAG03596.1	unnamed protein product [Tetraodon n...	812
gi	32186922	gb AAP72178.1	estrogen receptor alpha [Halichoeres...	810
gi	29169337	gb AAO66473.1	estrogen receptor alpha [Zoarces vivi...	809
gi	40217924	gb AAR82891.1	estrogen receptor alpha [Astatotilapi...	795
gi	2507414	sp P50240 ESR1_OREAU	Estrogen receptor (ER) (Estra...	783
gi	60101766	gb AAX13999.1	estrogen receptor alpha [Oryzias java...	783
gi	112982639	dbj BAF03498.1	estrogen receptor alpha [Kryptolebi...	781
gi	50293047	gb AAT72914.1	estrogen receptor alpha [Fundulus het...	780
gi	86278355	gb ABC88430.1	estrogen receptor alpha short form [K...	750
gi	12585224	sp P57753 ESR1_MICUN	Estrogen receptor (ER) (Estr...	746
gi	74422193	gb AAV25396.3	estrogen receptor alpha [Salmo salar...	694
gi	82582235	gb AAS92970.2	estrogen receptor alpha [Oncorhynchus...	692
gi	12643267	sp P16058 ESR1_ONCMY	Estrogen receptor (ER) (Estr...	692
gi	83026879	gb ABB96483.1	estrogen receptor alpha [Pseudolabrus...	686
gi	5101849	emb CAB45140.1	estrogen receptor [Oncorhynchus mykis...	680
gi	77020881	gb ABA60432.1	estrogen receptor alpha 2 [Oncorhynch...	651
gi	12061010	gb AAG48341.1 AF326201_1	estrogen receptor [Halicho...	629
gi	1706708	sp P50242 ESR1_SALSA	Estrogen receptor (ER) (Estra...	628
gi	103903	pir A37197	estrogen receptor - rainbow trout	609
gi	82409100	gb ABB73308.1	estrogen receptor alpha 1 [Oncorhynch...	595
gi	16118451	gb AAL12298.1	estrogen receptor alpha [Carassius au...	593
gi	56692005	emb CAD32175.1	putative estrogen receptor alpha [Ca...	592
gi	61097788	dbj BAD91035.1	estrogen receptor alpha [Rutilus rut...	585
gi	84619480	emb CAD67996.1	putative estrogen receptor alpha ...	583
gi	52789059	gb AAU87498.1	estrogen receptor alpha [Pimephale...	577
gi	23308675	ref NP_694491.1	estrogen receptor 1 [Danio rerio...	575
gi	95115499	gb ABF56051.1	estrogen receptor alpha [Spinibarbus...	575
gi	10944302	dbj BAB16893.1	estrogen receptor [Danio rerio]	574
gi	13872679	emb CAC37560.1	estrogen receptor alpha [Clarias gar...	561
gi	12230058	sp Q9YHZ7 ESR1_ICTPU	Estrogen receptor (ER) (Estr...	556
gi	38327072	gb AAR17610.1	estrogen receptor alpha-2 [Carassius...	554
gi	3818524	gb AAC69548.1	estrogen receptor type alpha [Ictaluru...	552
gi	83316220	gb ABC02394.1	estrogen receptor alpha [Hippoglossus...	526

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B. StAR

Sequences producing significant alignments:

				Score (Bits)	E Value	
gi	73915382	gb	AAZ92554.1	steroidogenic acute regulatory protein	498	2e-139
gi	109627819	gb	ABG34343.1	mitochondrial steroidogenic acute... protein	485	1e-135
gi	116090847	gb	ABJ56005.1	steroidogenic acute regulatory protein	448	2e-124
gi	20140240	sp	Q9DEB4	STAR ONCMY Steroidogenic acute regulatory protein	445	1e-123
gi	21362963	sp	Q9DE06	STAR SALFO Steroidogenic acute regulatory protein	442	1e-122
gi	29243194	dbj	BAC66210.1	steroidogenic acute regulatory protein	436	8e-121
gi	47209041	emb	CAF91743.1	unnamed protein product [Tetraodon nelsoni]	421	2e-116
gi	18859431	ref	NP_571738.1	steroidogenic acute regulatory protein	420	4e-116
gi	31323270	gb	AAP44111.1	steroidogenic acute regulatory protein	416	7e-115
gi	31323272	gb	AAP44112.1	steroidogenic acute regulatory protein	415	1e-114
gi	89474611	gb	ABD73012.1	steroidogenic acute regulatory protein	413	5e-114
gi	21362964	sp	Q9DG08	STAR XENLA Steroidogenic acute regulatory protein	378	2e-103
gi	63100238	gb	AAH95917.1	Unknown (protein for MGC:99207) [Xenopus laevis]	376	7e-103
gi	83405128	gb	AAI10789.1	MGC131332 protein [Xenopus laevis]	373	6e-102
gi	86276864	gb	ABC87916.1	steroidogenic acute regulatory protein	366	6e-100
gi	30584323	gb	AAP36410.1	Homo sapiens steroidogenic acute regulatory protein	345	2e-93
gi	57097785	ref	XP_532807.1	PREDICTED: similar to steroidogenic acute regulatory protein	345	2e-93
gi	60833148	gb	AAX37038.1	steroidogenic acute regulator [synthetic]	345	2e-93
gi	56243551	ref	NP_000340.2	steroidogenic acute regulator is... similar to steroidogenic acute regulatory protein	345	2e-93
gi	45382719	ref	NP_990017.1	steroidogenic acute regulator [GenBank:NP_990017.1]	345	2e-93
gi	114619693	ref	XP_001170357.1	PREDICTED: steroidogenic acute regulatory protein	345	2e-93
gi	727253	gb	AAC50141.1	steroidogenic acute regulatory protein	344	3e-93
gi	109086164	ref	XP_001090472.1	PREDICTED: similar to steroidogenic acute regulatory protein	342	1e-92
gi	116242803	sp	Q28918	STAR BOVIN Steroidogenic acute regulatory protein	340	6e-92
gi	3133060	emb	CAA76718.1	steroidogenic acute regulatory protein	337	6e-91
gi	1809329	gb	AAB41674.1	steroidogenic acute regulatory protein	336	7e-91
gi	27806397	ref	NP_776614.1	steroidogenic acute regulatory protein	335	1e-90
gi	3915025	sp	O46689	STAR HORSE Steroidogenic acute regulatory protein	335	2e-90
gi	57163979	ref	NP_001009243.1	steroidogenic acute regulatory protein	335	2e-90
gi	34582612	gb	AAQ76091.1	steroidogenic acute regulatory protein	335	2e-90
gi	7514090	pir	JC5386	steroidogenic acute regulatory protein -	334	4e-90
gi	56711334	ref	NP_998920.2	steroidogenic acute regulatory protein	333	4e-90
gi	45439548	gb	AAS64369.1	steroidogenic acute regulatory protein	333	8e-90
gi	2498964	sp	Q28996	STAR PIG Steroidogenic acute regulatory protein	332	1e-89

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C. Spermidine/spermine acetyl transferase

Sequences producing significant alignments:	Score (Bits)	E Value
gi 50344994 ref NP_001002169.1  hypothetical protein LOC43171...	272	5e-72
gi 71834540 ref NP_001025370.1  hypothetical protein LOC56570...	266	3e-70
gi 68404148 ref XP_696280.1  PREDICTED: similar to spermidine...	256	3e-67
gi 45383744 ref NP_989517.1  spermidine/spermine N1-acetyltra...	254	1e-66
gi 28188751 gb AAO16805.1  spermidine/spermine N1-acetyltrans...	251	9e-66

The entire sequence or any part of the sequence (at least 30 nucleotides in length) that is unique to the gene (or homolog) would provide correlative expression levels between control and exposed cells. Unique segments for genes can be determined by testing any segment via BLASTN to the entire genome sequence. A few segments may be specific for gene families (rather than the specific gene mentioned) and these segments would have lower correlative value – for example the DNA binding domain of all estrogen receptors (alpha, beta and gamma) is 95% identical – a sequence containing only this domain would not distinguish the three receptors from each other but would give an average value for their expression.

The Examiner asserts on page 7, beginning on line 16: ."the specification does not teach if these genes are observed in other species of fish and whether these genes provide the same expression in those species." In this case, all three of the receptors are up regulated by estrogen. The genes are expressed in other species of fish – as can be determined by BLASTP of the genes in Table 1. The inference is that these genes would be changed by estrogen or androgen in all fish species. Research we have performed in the last couple of years indicates that homologs of these

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same genes are changed in fathead minnows (a freshwater species) that are exposed to estrogens and androgens, among other genes.

Thus, by identifying the genes for largemouth bass or for sheepshead minnow that respond to stimulation by estrogens or androgens, either by increased or decreased expression, one can identify homologs in other fish species for which there is gene sequence information in the databases. Thus knowing that estrogen or androgen alter expression of this set of genes in largemouth bass and sheepshead minnow, one can reasonably expect that the same set of genes (or a subset of these genes) will respond in like manner in all fish species. The instant specification thus, allows one of ordinary skill in the art to identify homologs from any fish species without undue experimentation or otherwise.

8. Next, I will address some of the issues raised by the Examiner in the Office Action.

On page 8, beginning on line 3 of the Office Action: "This would require a large amount of inventive effort, with each of the many intervening steps, upon effective reduction to practice, not providing any guarantee of success in the succeeding steps."

Applicants disagree. There are microarrays commercially available for zebrafish and fathead minnow (each containing 60mer oligonucleotides) that could be used to determine response to estrogen or androgen. These experiments are straight forward and do generate data that corroborates the original statements that the genes in the instant application are altered by

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exposure to estrogens and androgens. It is now possible to get abundant EST sequences from any fish species that would allow similar interpretation.

On Page 9 line 16 of the Office Action: “..... The search of the SEQ ID NOS claimed indicates homology is not identical among species of fish.....:

Homology does not need to be identical among species of fish. One would identify the genes in other species by using the BLASTP program – as more sequence information is available, these genes would be identifiable in all species. The BLASTP program shows homology with mammalian species as well, thus the similarity would extend to all vertebrates. The homology does not need to be > 90% -- genes are normally found to be homologs because they share high homology in blocks of sequences that are highly conserved and may have low homology in other regions which are of less importance. Thus, one should be able to still identify the homologs and then design specific oligonucleotides based on the sequence of the homologs to test the species of interest.

On page 10, line 11: “if the scientist used the wrong probe sequence within the vitellogenin gene to make a gene chip, there is a high probability that no positive response would be observed on the chips.....”

The first step is to get sequence information for the gene of interest from any fish species. Then there is an algorithm that can be used to design the best 60'mer oligonucleotide for

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application to an array (or one could use the entire cDNA or the fragment obtained). For oligonucleotide probes there is a correlation of better probes with position on the cDNA towards the 3'-end. The algorithm to design oligonucleotide probes is now available on the Agilent web page. However the probes are designed, prior to use, it would be important to run the BLASTN program to determine that the sequence maps to only one gene.

9. I further state that all statements made herein are of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with my knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Nancy Denslow  
Dr. Nancy D. Denslow

Jan 4, 2007  
Date